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REVIEW

Application of DNA Markers for Breeding Carnations Resistant to Bacterial Wilt

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Abstract

Carnation bacterial wilt (CBW), caused by *Burkholderia caryophylli*, is one of the most damaging diseases affecting carnations in Japan. In this study, carnation breeding was conducted using CBW resistance derived from *Dianthus capitatus* ssp. *andrzejowskianus*. To map the genetic loci involved in resistance to CBW and develop the linked markers, the first molecular linkage map for carnation was constructed by using 134 progeny derived from a cross between 'Carnation Nou No. 1' (an interspecific hybrid of carnation and *D. capitatus*) and 'Pretty Favvare', a susceptible cultivar. The map consisted of 146 DNA markers and covered 16 linkage groups. QTL analysis identified a QTL with a significant effect and two QTLs with small effects. Evaluation of disease incidence in relation to the presence of the STS-WG44 marker, which is linked to a QTL with a large effect, revealed that marker-assisted selection (MAS) using STS-WG44 enables the tested population to be narrowed down by half. Repeated crossing and selection via both conventional disease screening and MAS led to successful development of the first CBW-resistant carnation cultivar, 'Karen Rouge'.

Discipline: Horticulture/Plant breeding
Additional key words: flower color, flower type, linkage map, QTL analysis, RAPD

Introduction

The carnation (*Dianthus caryophyllus* L.) is one of the major floricultural crops, not only in Japan but also worldwide. However, carnation production in Japan faces severe problems, such as the surge in carnations imported from overseas, e.g. Colombia and China, the slump in prices, and aging of growers. In 2010, imports accounted for 46.2% of the carnation market in Japan[30], the highest proportion of any ornamental plant. One of a range of strategies needed to break out of the present situation is "Breeding revolutionary new cultivars." Japanese growers must produce fresher and better quality carnations to compete with imports. Growers cannot compete with imports on quality using the same cultivars used by exporting countries because the climatic conditions, including day length and temperature, are more favorable for carnation production in exporting countries than in Japan. It is desirable to develop an original Japanese cultivar suitable for the Japanese climate and appealing to Japanese consumers. To achieve this, revolutionary techniques to produce new cultivars within a short period are needed.

The extensive study of plant genomes has sparked a revolution in molecular technique, while molecular markers have facilitated research into genetic variation at the DNA level. Marker-assisted selection (MAS), a method using DNA markers closely linked to relevant traits, holds great promise as a means of boosting the efficiency of the breeding line selection process. MAS enables accurate selection regardless of environmental factors and is possible even at the seedling stage, provided DNA can be extracted. MAS has been used in many crop breeding programs, and many commercial cultivars have been developed[40]. In ornamentals, expectations for the practical application of molecular markers have increased[4, 9, 32]. In recent years, floricultural research has focused on constructing genetic linkage maps and mapping genes for disease resistance, flower color, and other traits. This report reviews recent progress in the development of markers in carnation breeding for resistance to carnation bacterial wilt (CBW) by the NARO Institute of Floricultural Science (NIFS, Ibaraki, Japan).

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Breeding for CBW resistance

CBW, caused by *Burkholderia caryophylli* (Burkholder) Yabuuchi, Kasako, Oyaizu, Yano, Hotta, Hashimoto, Ezaki, and Arakawa, is one of the most damaging diseases affecting carnation cultivation in Japan, which results in serious crop losses, especially during summer. CBW was first found in Washington, USA, in 1941[14]. *B. caryophylli* also infects *Limonium sinuatum* Mill.[15], *Gypsophila paniculata* L.[19], and *Eustoma grandiflorum*[11] and has expanded into European countries. The main symptoms are sudden wilting, vascular discoloration, and the rotting of roots. Therefore, NIFS initiated a breeding program for CBW resistance in 1988. Onozaki et al. screened 277 cultivars and 70 wild *Dianthus* accessions to identify species resistant to CBW and found a highly resistant wild species, *D. capitatus* Balbis ex DC. ssp. *andrzejowskianus* Zapal (NIFS accession No. 14)[24, 25]. Subsequently, Onozaki et al. succeeded in introducing resistance from *D. capitatus* ssp. *andrzejowskianus* into carnations[23] and produced a new CBW-resistant line, 'Carnation Nou No. 1'[26]. However, this line has traits, such as small flower diameter and short stems, that make it unsuitable for cut flower use. To remove the genes responsible for the undesirable characteristics derived from *D. capitatus* ssp. *andrzejowskianus*, repeated crossing and selection for resistance to CBW was conducted.

Onozaki et al. used a cut-root soaking method for selecting progenies that were resistant to CBW[24, 25]. However, more than 3 months are required to determine the resistance of breeding materials in such inoculation assays and considerable labor is needed to screen the large amounts of material required. DNA markers are powerful alternative tools for disease resistance screening and expected to accelerate the breeding process.

Construction of a genetic linkage map in carnations

The construction of genetic linkage maps is a key step that serves several purposes, including studies of genome structure, map-based cloning of agriculturally valuable genes, and mapping of quantitative trait loci (QTL). Molecular markers linked to important agronomic traits can be used for MAS in breeding programs to improve selection efficiency. Genetic linkage maps have also been developed for some ornamentals, such as *Petunia*[31], roses[3, 5, 10, 13, 16, 33, 38, 48, 52], lilies[1], *Alstroemeria*[12], chrysanthemum[50, 51], and *Dendrobium*[41]. To map the genetic loci involved with resistance to CBW, the first molecular linkage map for carnations was constructed by using 134 progeny derived from a cross between 'Carnation Nou No. 1' and 'Pretty Favvare', a susceptible cultivar[42]. The map consisted of 124 loci derived from 137 random amplified polymorphic DNA (RAPD) and 9 simple sequence repeat (SSR) markers. These markers identified 16 linkage groups (LGs) covering 605.0 cM (Fig. 1).

This genetic linkage map in carnations revealed that the LGs differ from the haploid number ($x = 15$) and exposed the presence of several minor LGs. SSR are highly polymorphic, co-dominant, and transferable molecular markers suitable for use in MAS and as anchor points for comparing maps. Recently, Yagi et al. reported the first SSR-based genetic linkage map in carnations based on SSR-enriched genomic libraries and conducted expressed sequence tag (EST) analysis[47]. The map covered 843.6 cM, with an average distance of 6.5 cM between two loci but was not saturated. In carnations, a high-density saturated genomic map is needed to advance complex trait mapping and map-based cloning.

QTL analysis for CBW resistance

QTL analysis was applied to eight replication evaluations of resistance to CBW[42]. A QTL with a large effect (*Cbw1*, CBW resistance locus 1) was detected on LG 6, which accounted for 60.5% of the total phenotypic variance with an LOD score of 23.5. Two other QTL with small effect were detected on LG 2 (*Cbw2*) and 5 (*Cbw3*) with LOD scores of 2.3 and 2.9, respectively. Analysis of the results on the basis of different strains and inoculation seasons showed that while the positions and magnitudes of *Cbw2* and *Cbw3* varied, those of *Cbw1* remained unchanged. These results suggested that resistance to CBW derived from *D. capitatus* ssp. *andrzejowskianus* is related to one major and at least two minor genes (Fig. 2).

Although a previous study identified a marker tightly linked to *Cbw1*, to select only highly resistant lines to CBW, markers tightly linked to the minor QTLs (*Cbw2* and *Cbw3*) are needed[43]. QTL analysis using a high-density genetic linkage map will identify such markers and the gene responsible for CBW resistance by map-based cloning.

Evaluation of the efficacy of DNA markers in breeding lines resistant to CBW

To evaluate the utility of these markers in developing resistant lines, the presence of three identified markers, one located near each of the three QTLs, was investigated in backcross lines strongly resistant to CBW as a result of selection through resistance assays conducted to date[43]. The marker nearest *Cbw1* was WG44, which is the RAPD marker identified by Onozaki et al. by bulked segregant

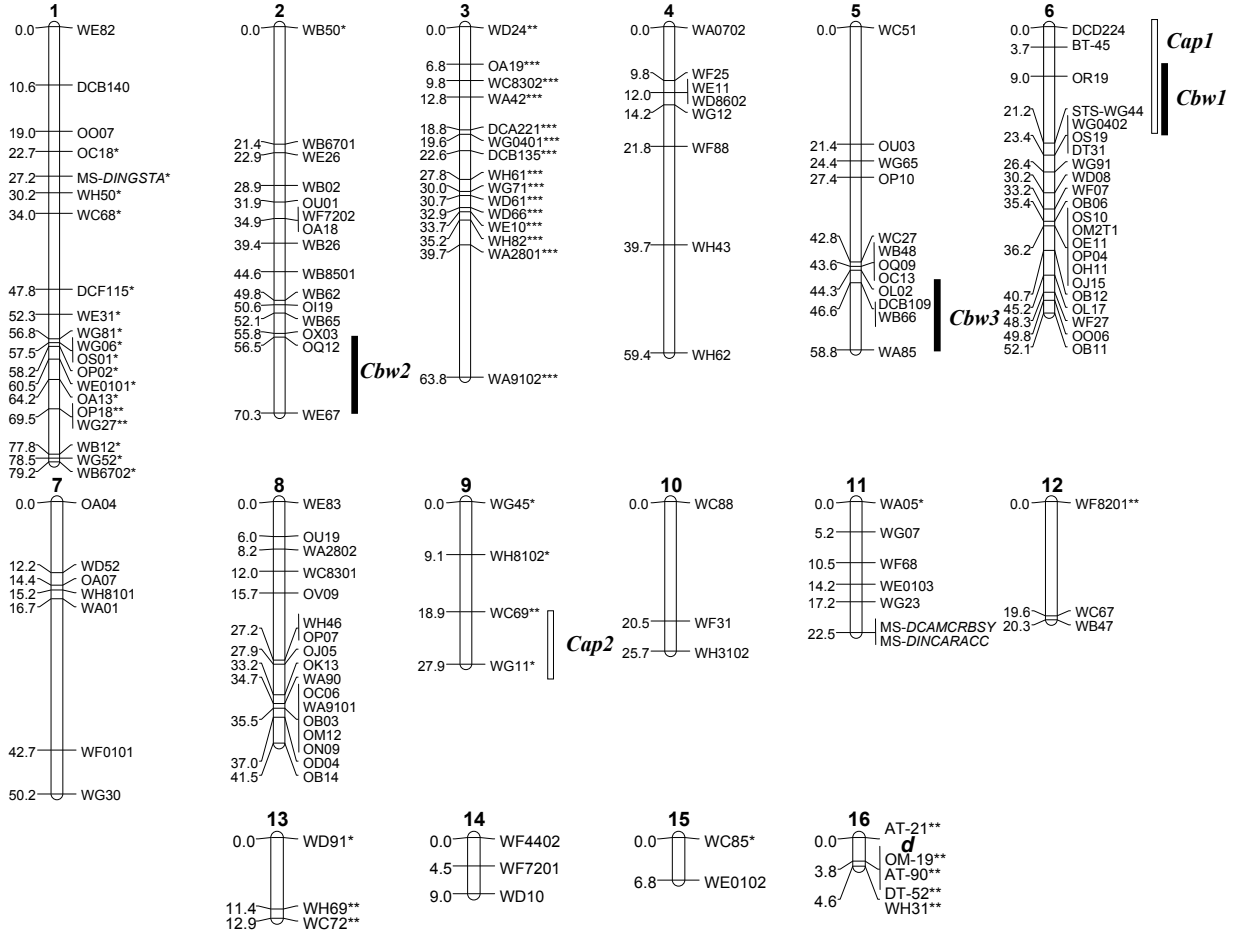

Fig. 1. Linkage map and QTL positions in carnations

Map distance (cM) is indicated on the left and locus names on the right. *, **, and *** indicate significant segregation distortions at 5, 1 and 0.1% levels, respectively, by chi-square tests. Bold and italic letters show the QTL regions for each trait (*Cbw*: CBW resistance locus, *Cap*: carnation anthocyanin pigmentation locus) and phenotypic marker *d*: single flower locus.

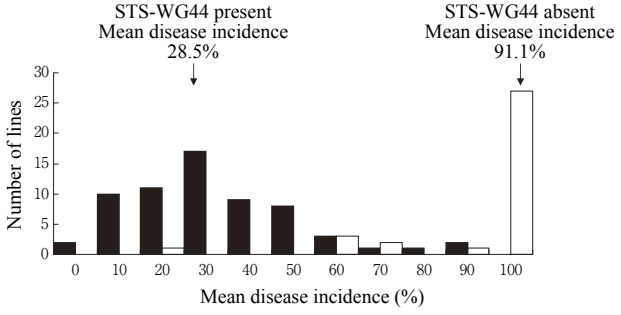

Fig. 2. STS-WG44 presence and disease incidence in actual breeding populations

■: STS-WG44 present (64 lines), □: STS-WG44 absent (34 lines).

analysis and from which they successfully developed a sequence-tagged site (STS) version (STS-WG44)[27]. An investigation into the presence of markers in 91 resistant backcross lines revealed that STS-WG44 was present in all lines up to the BC_4 generation (Table 1). However, OQ12, the marker on LG 2 linked to a QTL with a minor effect on resistance, was found in the F_1 generation only, namely 'Carnation Nou No. 1'. Moreover, its incidence declined in successive generations and was totally absent in the BC_4 generation (Table 1). Unlike OQ12, WB66, the LG 5 marker linked with a different QTL, which also has a minor effect on resistance, was present in all lines of the F_1 generation. However, it showed an equivalent tendency to OQ12, namely to decline in incidence in subsequent generations and become totally absent by BC_4 (Table 1), which suggests that these markers close to QTL and with

Table 1. Number and percentage of lines in resistant backcross lines carrying markers

Generation	Number of lines investigated	Number of lines carrying markers					
		Marker names (linkage group)					
		OQ12 (2)		WB66 (5)		STS-WG44 (6)	
F_1	7	1	(14.3)*	7	(100)	7	(100)
BC_1	18	7	(38.9)	14	(77.8)	18	(100)
$BC_1 \times BC_1$	6	2	(33.3)	4	(66.7)	6	(100)
BC_2	24	6	(25.0)	5	(20.8)	24	(100)
$BC_2 \times BC_2$	15	2	(13.3)	1	(6.7)	15	(100)
BC_3	15	2	(13.3)	1	(6.7)	15	(100)
BC_4	6	0	(0.0)	0	(0.0)	6	(100)

* Bracketed figures denote percentage carrying markers.

small effects on resistance are unsuitable for use in selecting resistant plants, whereas this experiment demonstrated the efficacy of the STS-WG44 marker in selecting resistant plants.

To ascertain whether STS-WG44 could also be used in actual breeding, disease incidence in relation to the presence of this marker in a total of 98 lines derived from crosses between susceptible and resistant backcross lines was investigated[43]. Investigation of disease incidence in 98 lines using the root-soaking inoculation method revealed a mean disease incidence ranging from 0 to 100%, showing large segregation for resistance (Fig. 2). The mean disease incidence was 28.5% in the 64 lines carrying STS-WG44 and 91.1% in the 34 lines lacking STS-WG44, revealing a significant difference of 62.6% depending on the presence or absence of STS-WG44. Of the 24 lines with a disease incidence of 20% or lower, 23 carried the STS-WG44 marker. These findings suggest that STS-WG44 is a selective marker that facilitates the narrowing of populations to those highly resistant for practical breeding.

By showing how STS-WG44 could be used as a selection marker to narrow carnation populations down to those with low mean disease incidence lines and strong CBW resistance, this study demonstrated the potential for MAS in the breeding of carnations.

Breeding of 'Karen Rouge'

Repeated crossing and disease screening were conducted to produce a new cultivar retaining the resistance derived from *D. capitatus* ssp. *andrzejowskianus*. In early breeding populations, many resistant lines had undesirable characteristics, such as few petals, dark flower color, or non-rigid stems, which were assumed to be derived from the wild species. Repeated crossing with more desirable cultivars gradually improved these unwanted characteristics in later generations. In 2004, MAS with the STS-WG44 marker was initiated. In 2010, using both traditional disease screening and MAS, Yagi et al. were able to develop a new red standard type cultivar, 'Karen Rouge', which had both high resistance and other traits desirable for ornamental use[46]. To my knowledge, 'Karen Rouge' is the first carnation cultivar produced using MAS technology.

Seven carnation cultivars or lines were used to improve the undesirable characteristics from *D. capitatus* ssp. *andrzejowskianus*. The mean disease incidence of 'Karen Rouge' was 7.1%, ranging from 0.0 to 19.0% across six tests. The susceptible carnation cultivars began to wilt 20 days after inoculation and most plants were dead within 49 days (Fig. 3). Conversely however, most of the 'Karen Rouge' plants survived the entire 91-day test. 'Karen Rouge' also showed high flower quality and adequate yields of cut flowers for commercial production in addition to high CBW resistance. 'Karen Rouge' is the progeny of a cross between the carnation cultivar 'Miracle Rouge', which has a long vase life[27] and the resistant line 4AZ31-5. The flower vase life of 'Karen Rouge' without chemical treatments is 13.1 days, which exceeds that of commercial cultivars (about 6–10 days).

Mapping of flower color and type

Using a population segregated for CBW resistance, Yagi et al. identified two QTLs (carnation anthocyanin pigmentation loci 1 [*Cap1*] and 2 [*Cap2*]) on LG 6 and 9, respectively, governing anthocyanin content in flower petals[44] (Fig. 1). *Cbw1* was located on the same linkage group

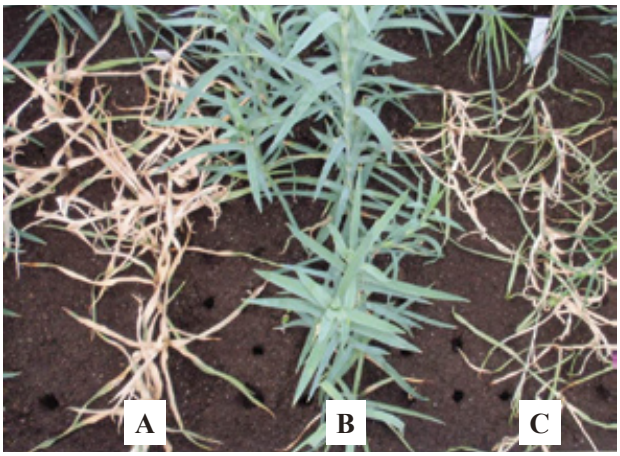

Fig. 3. Comparison of CBW resistance between cultivars 49 days after inoculation
A: 'Nora', B: 'Karen Rouge', C: 'Francesco'.

as *Cap1* at a distance of 15.6 cM. In the course of breeding for 'Karen Rouge', many resistant lines are observed to have purplish flowers in the initial breeding populations. These results could be explained by linkage between the major resistance gene and the QTL for anthocyanin pigmentation. Initial studies by Mehlquist and Geissman showed that a basic factor (S) controlled anthocyanin concentrations in carnation[22]. The structural genes encoding the enzymes involved in the anthocyanin biosynthesis pathway have been isolated, and relationships between the expression of these genes and anthocyanin synthesis have been elucidated in cyanic and white acyanic flowers[8, 20, 21, 49]. Mato et al. suggested that the structural genes for anthocyanin biosynthesis are controlled by regulatory genes, like *C1* or *P1* and *R* or *B* in maize, which share homology with myb proto-oncogenes and myc-like genes, respectively, and like *Del*, *Eluta*, and *Rosea* of *Antirrhinum majus*[21]. Larsen et al. suggested that the mutation of glutathione S-transferase (GST), which is involved in transporting anthocyanins to the vacuole, is responsible for the pale-anthocyanin coloration in carnation[18]. The QTL for anthocyanin content may be related to such genes that regulate the anthocyanin biosynthesis pathway or encode GST. The future mapping of genes involved in anthocyanin biosynthesis may allow us to determine whether any of these genes correspond to these QTL.

With respect to the flower type of carnation, although very little is known of the genetics of doubleness in carnation[2, 34], 80 years ago, Saunders suggested that the carnation flower phenotype was a monogenic trait and designated the locus involved as "D" (whereby the recessive homozygote allele [dd] is the single flower type, the heterozygote [Dd] is double, and the dominant homozygote DD is super double)[34]. Scovel et al. developed RAPD and RFLP markers linked to single flower locus d[35]. Onozaki et al. identified a single flower locus (*d*) on LG 16 in the abovementioned map and DNA markers linked to a recessive gene controlling a single flower type derived from *D. capitatus* ssp. *andrzejowskianus* (Fig. 1)[29]. Doubleness as a breeding characteristic is crucial in carnations[35], but all previous reports suggested that DNA markers were linked to the single flower locus (d). Co-dominat markers linked to doubleness (D), which can easily discriminate between the DD and Dd genotypes, are essential for carnations..

Utility of cultivar identification

Many carnation cultivars are registered in each country every year and registered varieties are protected by the Plant Variety Protection and Seed Act. In Japan, registered carnations that were illegally propagated have reportedly been imported[39]. Distinguishing the variety of and identifying agricultural and horticultural crops generally involves the use of morphological and physiological markers[37]. To counter these illegal actions, SSR markers for variety identification have been developed for carnations[17, 36, 37, 45].

Ornamentals, including carnations, have many mutants. It is extremely unlikely that such mutants can be discriminated from their original cultivars because they may result from a single point mutation and the chance of such a mutation involving one of the used SSR markers is minute[37]. A method to swiftly and unambiguously discriminate such mutations is therefore needed.

Conclusion

The first genetic linkage map for carnation and QTL analysis identified the STS-WG44 marker linked to a major QTL for CBW resistance. Repeated crossing and selection using both conventional disease screening and MAS using the STS-WG44 marker led to the successful development of the first CBW-resistant carnation cultivar, 'Karen Rouge'. MAS enables accurate selection regardless of environmental factors. MAS has been applied in breeding for resistance to black spot[6], but as far as I know, no development of a commercial rose cultivar through MAS has been reported. Therefore, I think that the production of 'Karen Rouge' by MAS represents an important advance in the breeding of ornamentals.

Next-generation sequencing technologies are now facilitating crop improvement[7]. The advent of second-generation sequencing platforms has made it possible to read billions of base sequence pairs in a single run or a limited number of runs. Next-generation sequencing can be

used to generate EST reads and subsequent transcript assemblies and identify genetic variations, accelerating the development of genomic resources from genic sequences. These technologies will change the situation species lacking significant genomic resources or with more complex (e.g. polyploid) genomic structures, such as ornamentals.

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